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Protocol

Method for culturing postnatal substantia nigra as an in vitro model of experimental Parkinson's disease

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Abstract

One of the most prevalent degenerative disorders of the nervous system is Parkinson's disease. The etiology of this disease is for the most part unknown, although it is posited to arise from an interaction of genetic and environmental factors. Although in vivo animal studies have been used to examine the effects of a number of Parkinson-inducing compounds, there is little information on reliable in vitro methodologies that can recapitulate the previously observed in vivo results. Here, we describe a method for generating mixed and chimeric neuron/glial cultures of postnatal substantia nigra (SN), independent of other monoaminergic nuclei in the ventral midbrain. Since many toxins do not affect regions of the midbrain except the SN, use of whole ventral midbrain from embryos can dilute any measurement of cell death. By specifically culturing ventrolateral midbrain containing the substantia nigra, one can more directly target the effects of dopaminergic toxins. In addition, this method can be used to test potential therapies for amelioration of Parkinson's disease.

Theme: Disorders of the nervous system

Topic: Degenerative disease: Parkinson's

Keywords: Substantia nigra; Parkinson's disease; Cell culture; *N*-methyl-4-phenyl-1,2,3,6 tetrahydropyridine hydrochloride (MPTP); Glia; Mice

1. Type of research

1. Development of a novel culture system to examine cell death following chemical insult.
2. Use of this system to examine drugs that might ameliorate cell death in experimental Parkinsonism.

2. Time required

2.1. Preparation of whole SN cultures

- Preparation of reagents, 1.5 h.
- Brain dissection and preparation of glial cultures, 4 h.
- Plating of whole SN onto laminin-coated slides prior to administration of drugs, 1 week.
- Administration of MPTP or 1-methyl-4-phenylpyridinium iodide (MPP+), 20 min/day for 2 days.
- Time of drug on culture prior to fixation, 7 days.

2.2. Preparation of chimeric cultures

- Preparation of reagents, 1.5 h.
- Brain dissection and preparation of glial cultures, 4 h.
- Culture of glial cells, 4 weeks before seeding neurons.
- Brain dissection of whole SN region, 4 h.
- Plating of whole SN onto glial feeder layer prior to administration of drugs, 1 week.
- Administration of MPTP or MPP+, 20 min/day for 2 days.
- Time of drug on culture prior to fixation, 7 days.

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3. Materials

3.1. Animals

Postnatal day 2–5 C57Bl/6 mice (Harlan, Indianapolis, IN) and/or Swiss-Webster (SWR) mice (Harlan).

3.2. Recipes for media

3.2.1. Bovine serum albumin (BSA) stock

1. Dissolve 100 mg/ml in Basal Medium Eagle (BME) and sterile filter.

3.2.2. Ovalbumin stock

1. Add 10 ml of BME to 1 g ovalbumin (Worthington Biochemical) and sterile filter.

3.2.3. Concentrated media additives (CMA) stock solution

For 100 ml: in 60 ml BME, add

1. 0.3 g L-proline (26 mM)
2. 0.3 g L-cystine (12.5 mM)
3. 0.1 g *p*-amino benzoic acid (7 mM)
4. 0.04 g vitamin B12 (0.29 mM)
5. 0.2 g inositol (11 mM)
6. 0.2 g choline chloride (14 mM)
7. 0.5 g fumaric acid (43 mM)
8. 8 mg coenzyme A (0.10 mM)
9. 0.2 mg D-biotin (0.008 mM)
10. 0.01 g D,L-6,8 thioctic acid
11. adjust pH to 7.4 with NaOH to get all components into solution. Add NaOH slowly while stirring to avoid excessive NaOH and high pH
12. increase volume to 100 ml with BME
13. freeze in aliquots and thaw immediately before use, avoid repeated freeze–thawing.

3.2.4. Feeding media

For 50 ml: to 45 ml BME, add

1. 0.25 ml of glutamine (1 mM final), (GIBCO #15039-027, 200 mM)
2. 180 mg glucose (20 mM final), (Sigma #G5767)
3. 0.25 ml CMA stock solution
4. 50 U/ml penicillin and 50 mg/ml streptomycin.

Bring to 50 ml with BME.

3.2.5. Feeding media complete with 2% rat serum

To 2.4 ml feeding media add

1. 400 μl insulin (250 mg/ml 10× stock, Sigma #I5500)
2. 1 ml transferrin (100 mg/ml stock, Sigma #T4515)
3. 1 μl sodium selenite (60 μM stock, Sigma #S1382)
4. 181 μl putrescine (2 mM stock, Sigma #P5780)
5. 180 μl rat serum (Harlan #4501).

Note: all stocks for this solution may be prepared, aliquoted, and stored frozen until use.

3.2.6. Dissociation media (DM)

For 1 liter combine:

1. 12.8 g sodium sulfate (90 mM final)
2. 5.2 g potassium sulfate (30 mM final)
3. 0.036 g calcium chloride.$2H_2O$ (0.25 mM final)
4. 1.18 g magnesium chloride.$6H_2O$ (5.8 mM final)
5. 1.8 g glucose anhydrous (10 mM final)
6. 0.238 g HEPES (1 mM final).

In deionized water, adjust pH to 7.4 with NaOH and sterile filter.

3.2.7. Plating media (PM)

For 100 ml

1. combine 1 ml stock BSA solution
2. 1 ml ovalbumin stock solution
3. 360 mg glucose (20 mM final)
4. 238 mg HEPES (10 mM final)
5. 0.5 ml CMA stock solution.

Bring to 100 ml with Basal Medium Eagle (BME), pH 7.4.

3.3. Special equipment

Nunc LabTek Permanox™ plates for culturing (Fisher #12-565-21).

4. Detailed procedures

4.1. Generation of SNpc cultures

1. Animals are placed on ice for 2–3 min to achieve an appropriate plane of anesthesia.
2. Animals are quickly decapitated and ~8–10 brains from P2–P5 C57BL/6 or SWR matings are removed and placed in a freshly prepared dissociation media (DM).
3. Brains are placed on their ventral surface and a slice of midbrain rostral to the cerebellum and caudal to the hippocampus is isolated (Fig. 1a). This removed brain slab is placed flat in DM and either the entire midbrain or the ventrolateral midbrain containing the substantia nigra is dissected and minced into small pieces (see Fig. 1b).

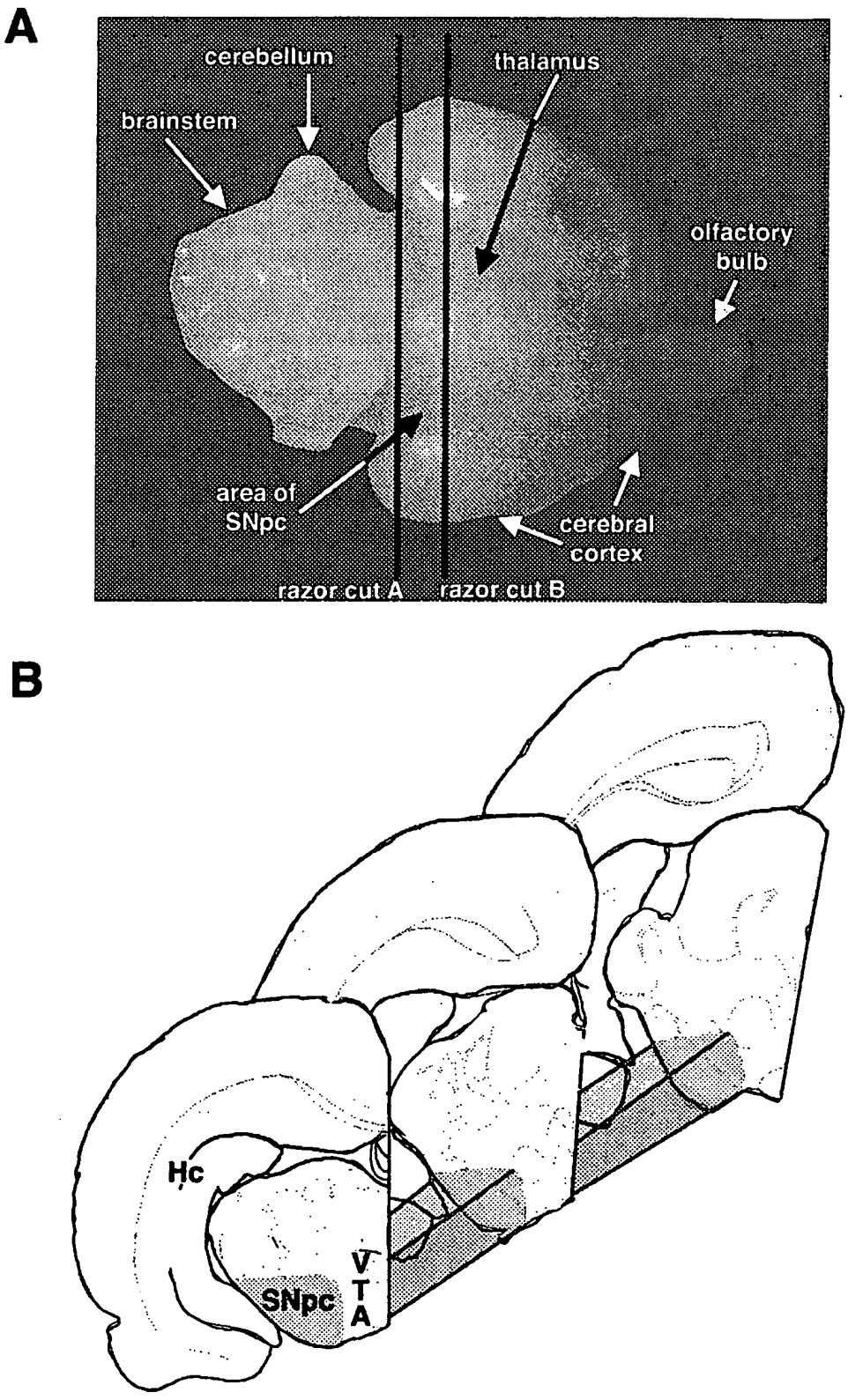

Fig. 1. Region of brain dissected to generate midbrain and substantia nigra cultures. (A) Prior to culturing, brains are removed and placed with the ventral surface of the brain upright. The first razor cut removes the posterior regions of the brain including the brainstem and cerebellum. A second razor cut is made to remove forebrain structures such as the thalamus, olfactory bulb as well as other forebrain structures not visible on the ventral surface such as the striatum and hippocampus. (B) Cartoon representation of the region of the brain dissected in the substantia nigra (SN) culture technique. Only the SN (grey highlights), and not the ventral tegmental area (VTA) or other midbrain dopaminergic and noradrenergic regions are dissected.

4. The minced substantia nigra or midbrain is then incubated in papain and DNAase (Dissociation Kit, Worthington Biochemical Corp., Freehold, NJ, follow kit instructions), for 30 min at 37 °C. A second incubation with fresh papain solution (30 min, 37 °C) is followed by three rinses in DM, and one rinse in plating media (PM).
5. Tissue is triturated in 5 ml PM and the cell suspension is added to 2 ml (1 ml of BSA stock and 1 ml ovalbumin stock).
6. The cell suspension is spun 1400 rev./min for 8 min and the pellet is then resuspended in 1.0 ml plating media containing 2% rat serum.
7. Once resuspended, cells are counted using trypan blue (0.4%) to determine cell viability.
8. Cells are adjusted to 1.2×10^6 cells/ml and plated at ~200,000 cells/cm^2 in Lab-Tek 4-well Permanox™ chamber slides that were previously coated with laminin (200 mg/ml, Collaborative Biomedical Products) and poly-D-lysine (200 mg/ml, Collaborative Biomedical Products) 1:1 (v:v) and rinsed once with deionized water. Cells are maintained in an incubator at 37 °C, 5% CO_2, and fed 2–3 times per week with feeding media complete with 2% rat serum (RS) by exchanging approximately one-fifth of the media (100 μl).

4.2. Chimeric neuron/glial cultures

1. SNpc cells plated on pre-plated SNpc glia are produced using a variation of the previously described methods. SNpc glial feeder layers from P2–5 C57BL/6 or SWR mice are produced using the above described method, but cells are plated at 20,000–50,000 cells/cm^2 and fed with plating media containing 2% rat serum and 8% fetal bovine serum (FBS) to promote glial proliferation and neuronal death.
2. For astrocyte cultures, this is the final step.
3. To generate mixed neuron/glial cultures, 3–4 weeks after the initial SNpc cells are plated, the glial feeder layers are rinsed once with plating media without serum, and 0.5 ml of plating media with 2% rat serum (RS) are added.
4. SNpc cells from C57BL/6 or SWR mice are isolated (as above) and plated at 250,000 cells/well on the previously generated glial feeding layers.
5. Twenty-four hours after plating of neurons onto the glial feeder layers, the cultures are fed with feeding media complete with 2% RS by an xchang of approximately one-fifth of the media (100 μl) and cytosine β-D-arabinofuranoside (Ara-C, 2 μM) to prevent glial proliferation of the freshly plated cells. Thereafter, Ara-C (10 μM, final concentration) is added at each feeding.

4.3. MPTP treatment

1. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Sigma Chemical Co., St. Louis, MO) is added to cultures 7–10 days after the neurons are plated onto glia. The MPTP is prepared by dissolving MPTP in feeding media for a 1 mM stock, then diluting to a 1 μM stock with feeding media complete with 2% rat serum and adding this directly to the cultures. The final concentration of the MPTP in media is 50 nM. Two feedings of MPTP, in 2 days, are necessary to achieve the desired toxic effect [8].
2. Seven days after MPTP is added, the cultures are rinsed 3× with TBS, fixed in 4% buffered paraformaldehyde for 10 min, and rinsed 3× with TBS.
3. MPTP/MPP+ is a potent neurotoxin and should be handled with care. Gloves and a mask should be worn while handling these products. Refuse from these substances should be mixed with a combustible solvent and burned in a chemical incinerator equipped with an afterburner and scrubber.

4.4. Identification of SN cells

1. To determine the number of SN cells, cultures are immunostained for expression of tyrosine hydroxylase (Fig. 2). Firstly, endogenous peroxidase activity is quenched by rinsing with 0.3% hydrogen peroxide in methanol in 1× TBS for 2×15 min. Cells are permeabolized with 0.1% Triton X-100, 5% goat serum

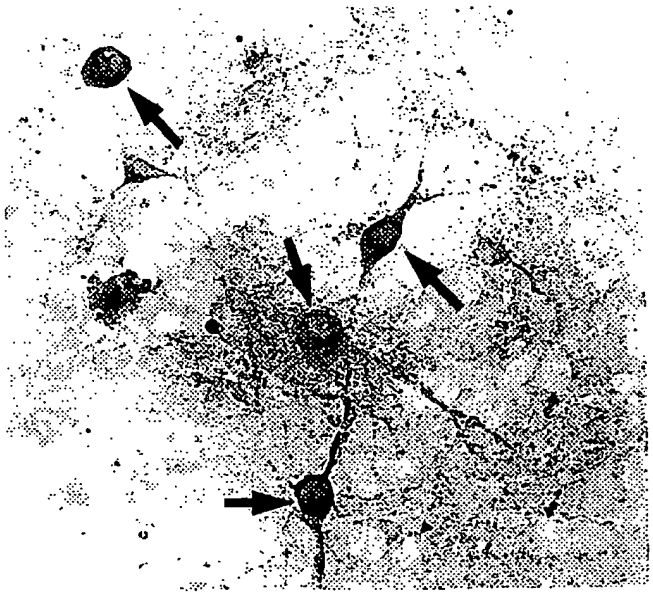

Fig. 2. Appearance of substantia nigra neurons after tyrosine hydroxylase immunostaining. Cells isolated from the SN are round to oval in shape with a clear nucleus (arrows). These cells may appear bipolar or multipolar depending on the number of processes emanating from the soma. The large unstained cells with clear nuclei under the SN neurons (arrowheads) are cells of the glial mat.

in TBS for 2×15 min. Cultures are covered in 400 μl of a polyclonal antibody directed against tyrosine hydroxylase (Eugene Tech International, Ramsey, NJ or Pel-Freeze, Burlingame, CA: each 1:500) and incubated in this solution overnight at 4 °C. The next day, cultures are rinsed three times with TBS followed by application of secondary antibody (goat α-rabbit) and amplification with Avidin–Biotin (ABC Elite Peroxidase kit, Vector Labs, Burlingame, CA). Final visualization of the immunopositive neurons is made using diaminobenzidine (DAB kit, Vector Labs) as a chromagen.

2. All TH-positive cells having the previously described characteristics of SNpc neurons (TH-positive cytoplasm surrounding a pale unstained nucleus) [2] from each culture are counted at a magnification of 200×.

Note: to control for variability between cultures, all MPTP treatments are performed on matched cultures present on one single slide. Once cells are counted in each well, the number of TH-positive cells in the MPTP-treated cultures from each genotype are compared to the adjacent non-treated culture. Since an identical number of cells generated from the same brains were plated on a single slide, these cultures are directly compared to determine the percent cell loss following MPTP. The percent cell loss following MPTP are then pooled from all C57Bl/6, SWR or mixed cultures.

5. Results

Both methods yield viable cultures, with a large number of healthy neurons (Fig. 3). We estimate from initial counts of the SN in vivo [2], that through the culturing procedure, ~50% of the postnatal neurons die. Thus to have the necessary number of neurons for an experiment, one needs to dissect ~8–10 early postnatal brains of each genotype.

MPTP and MPP+ administration to SN cells in vitro result in the loss of ~50% of the C57Bl/6 neurons and 2–3% of the SWR neurons [8]. The percent loss of neurons in the in vitro system is slightly lower than the cell loss seen in each of these strains in vivo [2]. The slightly lower cell loss is attributed to the presence of MPTP-insensitive neurons that are present in the ventrolateral dissection of the midbrain [8].

6. Discussion

6.1. Overall assessment of the procedure

1. This protocol details an in vitro cell system that can be used to examine the protective or detrimental effects of small molecules on neuronal death in the MPTP model of Parkinson's disease. The benefit of this procedure over other published methods is that the tissue(s) used in this procedure include only the specific cells lost in Parkinson's disease rather than a large number of unaffected cells (Fig. 3) [3–7]. In addition, this system can be modified to examine, in vitro, cells affected in other degenerative or developmental processes. The advantage of the preplated glial mat used in the protocol is that it makes it possible to increase neuronal survival when there are few neurons available in the sample or the neurons are difficult to culture and maintain in vitro.
2. The methods to preferentially grow neurons or glia, and the generation of chimeric cultures from each of these cell lineages can be used to investigate cell–cell interactions in a number of developmental or neurological disease models.
3. The major drawback of the technique is that there is a comparatively low yield of cells relative to the amount of time needed to produce the samples. The labor intensive aspect of the technique may be overcome by the ability to obtain answers to specific questions about degenerative processes in the nervous system.

6.2. Trouble shooting

Consideration of certain parts of the protocol may help to avoid problems later.

1. Plating surface: 4-well Labtek chamber slides (Permanox™) provide enough surface area to support cells in numbers suitable for analysis. The surface area of 4-well slides yields more consistent coverage of neurons/area compared to 8-well slides in which surface tension of the small volume may cause neurons to be concentrated around the edges of the well. In addition, plated cells adhere better and more uniformly when the laminin/poly-D-lysine coating is applied roughly within 24 h of use and rinsed off immediately before plating cells.
2. The purified glial mat reaches confluence at ~3–4 weeks for substantia nigra tissue, and this appears to be the best time to plate neurons. If neurons are plated prior to this time, the glia may not be confluent or some unwanted neurons may still be present. Glial mats generated from substantia nigra cultures that are allowed to age beyond 4 weeks may begin to deteriorate. This time table can be adjusted based upon the brain region harvested to produce the glial mat as well as the initial number of cells plated prior to the glia growing to confluence.
3. In the immunohistochemistry section of the protocol, a Tris-based buffer, such as TBS, is used rather than a phosphate buffered solution (PBS), since PBS after the

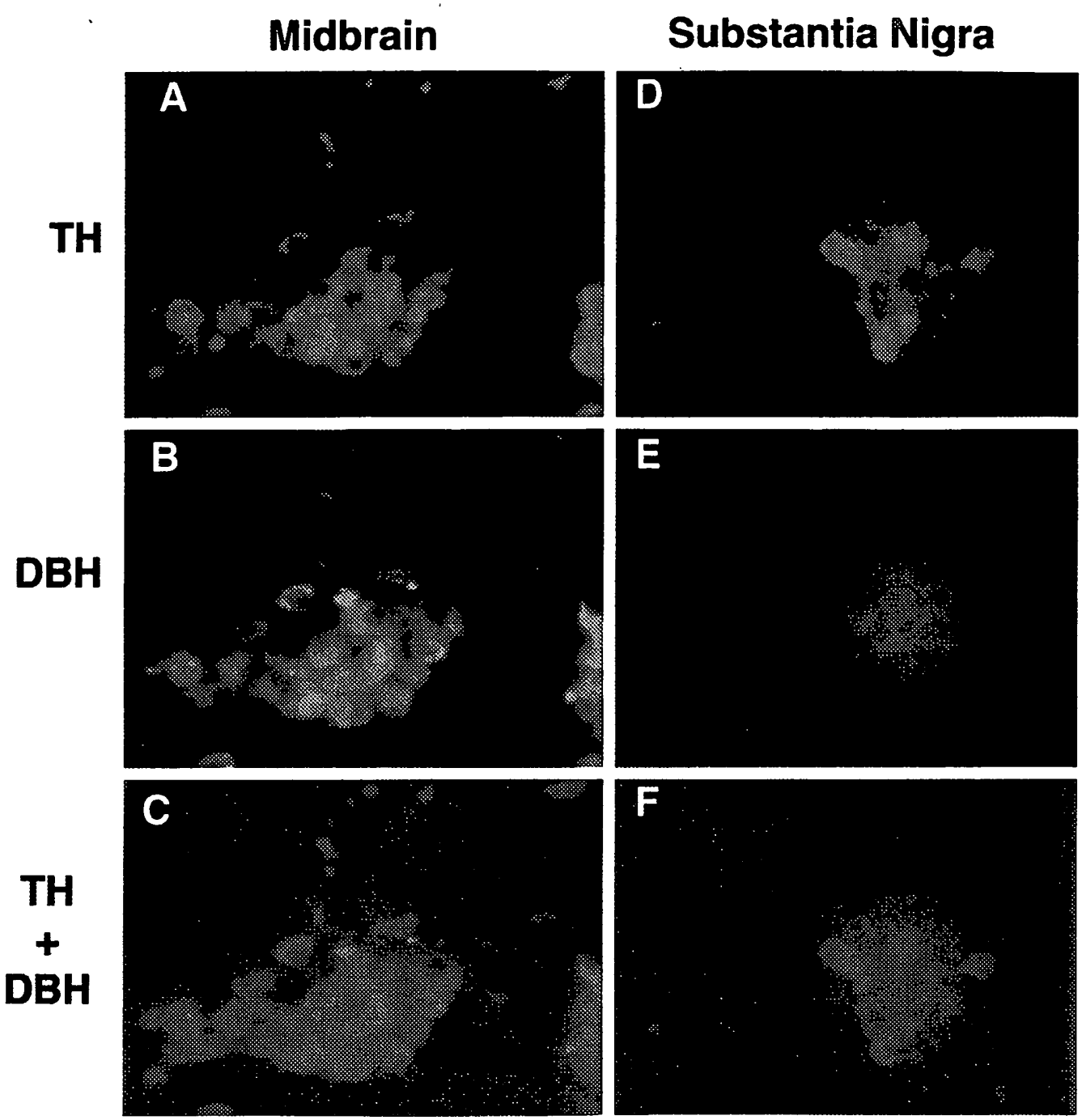

Fig. 3. Identification of monoaminergic cells in midbrain and substantia nigra cultures. A cluster of cultured cells from whole midbrain cultures are immunopositive for (A) tyrosine hydroxylase (TH), present in all monoaminergic neurons and (B) dopamine β-hydroxylase (DBH) which is specific for noradrenergic neurons. Dopaminergic cells will contain TH, but not DBH. A merged image of A and B shows that TH and DBH are colocalized. In substantia nigra cultures, neurons are immunopositive for (D) TH, but not for (E) DBH. A merged image (F) shows that these TH cells do not express DBH.

addition of MPTP or MPP caused crystals to form in the well, making cell counts and microscopy difficult.

6.3. Additional information

Support for this protocol can be found in Cardozo et al. [1], from which the basis for this protocol was modified.

7. Quick procedure

1. Remove brains from P2–P5 mice.
2. Dissect midbrain or SN from brain.
3. Make single cell suspension of neurons and glia with papain incubation.

4. Spin cell suspension to pellet, resuspend cells and check for viability.
5. Plate cells at 200,000 cells/cm^2 in Labtek 4-well Permanox™ plates.

For chimeric cultures.

1. Follow steps through step 4 (above).
2. Plate cells at 20,000–50,000 cells/cm^2 in Labtek 4-well Permanox™ plates.
3. One month later, repeat steps 1–4 and plate 250,000 cells/cm^2 on top of preplated glial layer.
4. Treat with ARA-C to stop glial proliferation.

Drug treatment.

1. Treat cultures two times with 50 nM MPTP, each application spaced 1 day apart.
2. Fix cells after 7 days.

Visualizing neurons.

1. Immunostain fixed cultures with antibodies directed against tyrosine hydroxylase (1:500), fix and coverslip.
2. Count neurons in each well.

8. Essential references

Original papers: [1], [8].

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